(16) Keratitis

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Brief Communication

TNF-α production in the cornea in response to *Pseudomonas aeruginosa* challenge

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Summary *Pseudomonas aeruginosa* can cause ulcerative bacterial keratitis or contact lens-induced acute red eye (CLARE) in humans. The present study used a mouse model of ocular infection and inflammation to examine the relationship between TNF-α and inflammation in the cornea in response to challenge with either a strain of *P. aeruginosa* causing keratitis or a CLARE strain. Constitutive TNF-α mRNA was detected in the epithelium, mainly towards the periphery. After infection with the keratitis-inducing strain (6294), TNF-α expression was elevated four-fold by 24 h post-challenge. No detectable induction of TNF-α mRNA was seen with CLARE strain (Paer1) challenge at any time point. The TNF-α protein production detected by ELISA showed a corresponding pattern to the mRNA expression, which also correlated with pathological changes. These results suggest that invasive strains of *P. aeruginosa* create greater pathological changes as a result of elevated TNF-α production, which contributes to inflammation during keratitis *in vivo*.

Key words: cornea, inflammation, *P. aeruginosa*, TNF-α.

Introduction

Pseudomonas aeruginosa is the bacterial pathogen most commonly associated with microbial keratitis during contact lens wear,[1] and is also frequently associated with contact lens-induced acute red eye (CLARE).[2] CLARE is a non-infectious keratitis resulting from *Pseudomonas* colonization of contact lenses.[2] The release of pro-inflammatory cytokines such as TNF-α[3] and of specific chemotactic cytokines at the site of bacterial challenge regulates the processes of inflammation and leucocyte recruitment. Very little is known about the nature of the inflammatory mediators produced in the cornea, but the host inflammatory response is important in the outcome of keratitis. While during Gram-negative systemic infections, TNF-α is thought to act as an obligate precursor for further cytokine responses,[4] its role in corneal infection is not well defined.

A better knowledge of the host response during bacterial keratitis may lead to improved strategies for the prevention and management of these potentially serious ocular conditions. In experiments reported here, we examined the production of TNF-α in the cornea in response to challenge with two different strains of *P. aeruginosa*.

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Materials and Methods

Infection of animals

Adult BALB/c mice were challenged with corneal scarification and instillation of 2×10^6 cfu of *P. aeruginosa* strain 6294 (isolated from microbial keratitis) or Paer1 (isolated from CLARE) in 5-μL suspension, or PBS under anaesthesia with Avertin as previously described.[5] The right eye of each animal served as a control. All protocols were approved by the Animal Care and Ethics Committee, University of Sydney, New South Wales, Australia. It has been demonstrated recently that the mouse model of bacterial keratitis can be used to mimic CLARE.[5] Strain Paer1 was chosen as this is the only strain isolated from CLARE that has been characterized *in vitro* and *in vivo*.[5] Strain 6294 was chosen as a representative strain of invasive *P. aeruginosa*.[6]

In situ *hybridization and ELISA*

Mice were killed at 0, 1.5, 4 and 24 h post-challenge. Formalin-fixed, paraffin-embedded corneal tissues were cut at 5 μm. The sections were dewaxed, rehydrated and target mRNA was exposed by digestion with 10 μg/mL proteinase K at 37°C for 20 min.[7] Corneal whole mounts on slides were fixed in 4% paraformaldehyde in PBS for 10 min, and rinsed in PBS. Corneas were digested with Pronase E.[8] A murine TNF-α cDNA was provided by Dr Andrew Lloyd, School of Pathology, University of New South Wales. The TNF-α riboprobe (sense and antisense) was prepared as previously described, and labelled with either digoxigenin[7] or 35-S[9] nucleotide mixtures (Boehringer Mannheim, Sydney, NSW, Australia). Sections were hybridized with either the sense (control) or antisense dig-labelled probe in a hybridization buffer,[7] or whole mounted corneas were hybridized with sense or antisense 35-S-labelled probes. Slides were incubated in a slide thermocycler at 37°C for 18 h, and washed in Tris-buffered saline (pH 7.5). The slides were incubated with

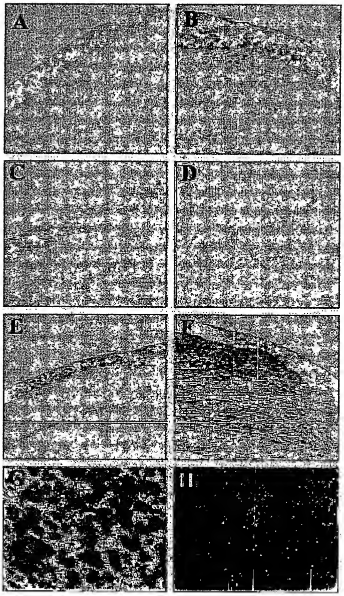

Figure 1 Photomicrograph showing detection of TNF-α mRNA in cornea challenged with *P. aeruginosa*. The TNF-α mRNA expression in the cornea is shown at (A) 0 h and 24 h in response to (B) 6294 strain and (C) strain Paer1 challenge. Sense control (challenged with 6294 and killed after 4 h) shows (D) no detectable signal. The pathological changes in the cornea induced by strains (E) Paer1 and (F) 6294 at 24 h post-challenge are demonstrated by haematoxylin and eosin staining. The expression of TNF-α mRNA in the cornea is confirmed by isotopic *in situ* hybridization in (G) bright field and (H) dark field conditions of Paer1-challenged corneas. These are typical results of three replicate experiments with at least three mice per group. All photographs are 200× magnification. Apparent differences in magnification are due to increasing oedema of the cornea during infection with 6294.

antidigoxigenin antibody conjugated with alkaline phosphatase for 1 h at 24°C. The colour development was according to the manufacturer's instructions (Boehringer Mannheim). For 35-S-labelled sections, the procedures were performed as described.[8,9]

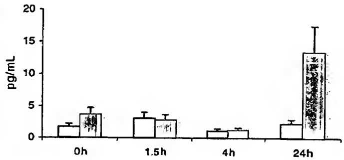

Figure 2 Tumour necrosis factor-α protein levels in the eye in response to *P. aeruginosa* infection using Paer1 (□) or 6294 (□) strains of bacteria determined by ELISA assay. The data represent means of ELISA results and the vertical bars are standard errors of the mean.

Samples for ELISA were collected by killing three mice at each point and enucleating three left or right eyes. The eyes were homogenized in 1.0 mL PBS containing 0.05% v/v Triton-X 100 at 4°C. The supernatant was collected and immediately frozen at −70°C until required. The ELISA kits for murine TNF-α (R & D Systems, Sydney, NSW, Australia) were used according to the manufacturer's instructions.

Results and Discussion

Histological morphology was compromised because of proteinase digestion to expose mRNA, and all digoxigenin-labelled sections were not counterstained. Sections hybridized with the control probe showed no positive signal (Fig. 1D). Background levels of TNF-α mRNA were observed in corneal epithelium at 0 h post-challenge (Fig. 1A) and no significant induction was seen at 1.5 and 4 h (data not shown). At 24 h post-challenge, cells in the epithelium were positively stained; in the periphery and to a lesser extent in the central stroma, some infiltrating inflammatory cells were positive for TNF-α mRNA (Fig. 1B). Little induction of TNF-α mRNA expression was detected during corneal challenge with Paer1 at any time point. Figure 1(C) shows the cornea 24 h after initial Paer1 application, and the levels of positive staining were similar to time zero (Fig. 1A). To confirm the results of the digoxigenin-labelled detection system, the 35-S-labelled probe was used on whole mounted corneas to avoid any sampling errors arising from sectioning. Positive signals were detected mainly at the periphery of the corneal epithelium in eyes challenged with either Paer1 (Fig. 1G,H) or 6294 (data not shown), which is consistent with findings in the digoxigenin system. Negative controls demonstrated a low level of background staining only. Pathological changes were evaluated by haematoxylin and eosin staining (Fig. 1E,F).

Consistent with *in situ* hybridization results, ELISA assay revealed that constitutive background levels of TNF-α production were detected in the cornea at all time points in response to strain Paer1 challenge (Fig. 2). However, there was no increased TNF-α production in the eye in response to strain 6294 challenge at 0, 1 and 4 h, but approximately a four-fold (significant, $P < 0.05$) induction was noted at 24 h.

Our findings are supported by the work of Svanborg *et al.* who have shown that in a compartmentalized Gram-negative

infection of the mucosal epithelium of the bladder, there was no significant TNF-α produced early in the infectious process, while there is a large and rapid IL-6 and IL-8 response as early as 30 min post-challenge.[4] Others examining the ocular response to Gram-negative infection in whole eye homogenates also found induction of TNF-α above baseline levels between 1 and 3 days post-infection.[10] In a systemic response to Gram-negative infection, induction of TNF-α would be expected after 60–120 min.[11] There has been a recent report of TNF-α mRNA up-regulation during *P. aeruginosa* keratitis as early as 6 h after infection by a ribonuclease protection assay, which may be a reflection of the different techniques.[12] It may also be due to genetic factors determining the outcome of cytokine production.[8] It is possible that the absence of TNF-α during the early stages of localized Gram-negative infection at mucosal surfaces is a part of the mucosal cytokine response, which differs from that observed during systemic Gram-negative infection, where it is thought to be an obligate precursor for further cytokine responses.

Acknowledgements

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References

1 Schein OD, Ormerod LD, Barraquer E *et al. Cornea* 1989; **8**: 281–5.
2 Holden BA, La Hood D, Grant T *et al. CLAO J.* 1995; **22**: 47–52.
3 Akira S, Hirano T, Taga T, Kishimoto T. *FASEB J.* 1990; **4**: 2860–7.
4 Svanborg C, Agace W, Hedges S, Lindstedt R, Svensson ML. *Ann. N.Y. Acad. Sci.* 1994; **730**: 162–81.
5 Cole N, Willcox MDP, Fleiszig SMJ *et al. Exp. Eye Res.* 1998; **17**: 730–5.
6 Fleiszig SMJTS, Zaidi MJ, Preston M *et al. Infect. Immun.* 1996; **64**: 2288–94.
7 Cole N, Willcox MDP, Tout S *et al.* In: Husband A, Beagley K *et al.* (eds). *Mucosal Solutions: Advances in Mucosal Immunology.* Sydney: University of Sydney, 1998; 327–35.
8 Zhou Y-F, Bao S, Rothwell TLW, Husband AJ. *Eur. J. Immunol.* 1996; **26**: 2133–9.
9 Bao S, Goldstone S, Husband AJ. *Immunology* 1993; **80**: 666–70.
10 Kernacki KA, Berk RS. *J. Ocul. Pharmacol.* 1994; **10**: 281–8.
11 Waage A, Brandtzaeg P, Halstensen A, Kierulf P, Espevik T. *J. Exp. Med.* 1989; **169**: 333–8.
12 Kernacki KA, Goebel DJ, Poosch MS, Hazlett LD. *Infect. Immun.* 1998; **66**: 376–9.